My comments:

I think the experiment is well thought out and sets out to examine an interesting question; however, I'm not currently convinced the experimental setup does allow you to examine a "progressive nitrogen limitation" hypothesis. I'm happy to be corrected on this point, but if I've followed, the experiment setup is more like a gradient of treatments, which is being used as a substitute for a longer-term change in nitrogen availability. I'm sure I missed it but I could not determine how long the experiment was being run for (?), in the absence of this information, I'm assuming a relatively short growth cycle. My view is that although there is nothing wrong with the experiments setup in the paper, you are testing a fundamentally different question from the one reflected in Luo's hypothesis about a change in nitrogen availability with time (where time is some extended period - in the FACE experiments - years). You can of course make a space-for-time argument, but I think you need to walk the reader through this idea more than you have and also note that these are rarely the same thing. Regardless of whether you agree or disagree with me here, a little more detail to support the experimental motivations is warranted (see comments below).

Overall, I found the manuscript easy to read, but I wonder if in revision you could work on making the link between the hypothesis, experiments and the results/discussion a little more compelling. The results section is quite dense in its reporting style and I think as a reader it can be hard to make a link to the starting questions as you read through it. One way to do this might be to streamline some of the presented material, for example, I was unclear why you report changes in Rd25, Chl or things on both an area and mass basis (could one go in the supplement?). Similarly, all the results have an identical format - this is not a problem (to be very clear), but I wonder if there is another way to report some of the results that much better capture the reader's interest - or help them better follow changes between figures. Can I also suggest you add a few lines to the methods to explain the role of inoculation, why such granularity on the nitrogen treatments, and why you opted for one very high CO2 manipulation. In the Discussion, I recommend a separate limitations subheading - currently the discussion about roots growth potentially being limited by pot size is buried in with modelling implications. I agreed with R2 that some of the literature review was a little selective, given the pitching around "cost of acquisition", citing at least one of the FUN papers is warranted. Perhaps a more explicit link could be made when discussing the modelling implications and considering whether the FUN model was able to capture your experimental findings.

Sincerely,

Martin De Kauwe

\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*\*  
In any correspondence regarding this manuscript, please include the manuscript reference number and copy the correspondence to New Phytologist Central Office ([np-managinged@lancaster.ac.uk](mailto:np-managinged@lancaster.ac.uk)).  
  
Decision: Reject  
  
Referee: 1  
  
Comments to the Author  
The paper reports a growth-chamber experiment that examined the effects of rhizobial inoculation and nitrogen fertilization on leaf physiological and whole-plant growth responses of soybean to high atmosphere CO2. Plants were grown for 7 weeks in the peat moss and sand mix irrigated with modified Hoagland solutions, The study tackles an important issue of plant response/adaptation to future climate change scenarios, and provides some interesting results. It fits well within the scope of the journal.  
  
General comments  
1.      My main concern is the CO2 levels; 420 ppm as an ambient control and 1000 ppm as elevated CO2. I am wondering why 1000 ppm was used as the high CO2 treatment; the reason should be given in the M&M. The majority of FACE studies have chosen 500-550 ppm while controlled-environment studies have used around 700 ppm as elevated CO2 levels. For some plant species, the CO2 levels for the maximum growth and optimal metabolism are around 550-700 ppm.  Have you done a preliminary experiment if 1000 ppm is still at an optimal range for soybean? If 1000 ppm is too high for soybean, then the conclusion you've drawn need be justified. It would be great if you had included another CO2 level 600 ppm in the treatments.

The purpose of this experiment was not to fall within the range of what is considered “optimal” for soybean. Elevated CO2 concentrations will inevitably take

2.      Results: Suggest to simplify the text based on the stats; when interactions are not significant, just talk the main effect, if interactions are significant, just talk about the interactions. “(p<0.001 in all cases; Table 1)” may be simplified to “(p<0.001; Table 1)”, and “were stronger under aCO2 than eCO2 (Tukey test of the nitrogen fertilization slope between CO2 treatments: p<0.05)” to “were stronger (higher slope) under aCO2 than eCO2 (p<0.05).

3.      At the beginning of a sentence, the use of an abbreviation should be avoided. Please change eCO2 to Elevated CO2 at the beginning of sentences throughout.

Changed accordingly  
  
Specific comments  
L113: over-investment?

Done

L181: Did the pots have drainage holes? As the use of water/nutrients differed between the treatments, was there any possibility some treatments might accumulate ions or over-saturated while others depleted them outside optimal ranges during the experiment? You may briefly explain if this was not the case.

Done

L314: Were the inoculated plants all nodulated across the N treatments? You may state the observation somewhere, probably in the results.

Yes

Tables 1-3: Change \* to multiply sign x for interactions. For example, I\*N should be I × N. Please also check the df. For ANOVA, df = n-1… but I am unfamiliar with your stats method. The same suggestions are applied to the supplementary materials.

Changed. Df is correctly reported for the Type II Walds Chi-square  
  
Supplementary materials  
Table S1: It appears that the cations were not fully balanced across the 9 N treatments. In addition, CaCO3 is insoluble and you cannot make 1 M. People generally use CaCl2 to balance Ca.

Cations were fully balanced across the 9 N treatments and CaCO3 is a standard protocol used in Hoagland’s solutions. We can, and did, make 1 M CaCO3  
  
Referee: 2  
  
Comments to the Author  
Review of Manuscript NPH-MS-2024-46044: "Nitrogen demand, availability, and acquisition strategy control plant responses to elevated CO2 at different scales"  
  
The manuscript titled "Nitrogen demand, availability, and acquisition strategy control plant responses to elevated CO2 at different scales" by Evan A. Perkowski, Ezinwanne Ezekannagha, and Nicholas G. Smith presents a compelling study aimed at unraveling the intricate dynamics between nitrogen (N) demand, availability, and plant acquisition strategies under elevated CO2 conditions. Utilizing Glycine max seedlings as a model, the study employs a full-factorial experimental design to explore these interactions, offering insightful contributions to the discourse on plant adaptation strategies in the face of climatic changes.  
  
The manuscript makes a significant contribution to understanding the complex interplay between nitrogen dynamics and plant responses to elevated CO2. With the suggested revisions below, including a more in-depth literature review, I believe it will offer valuable insights to the scientific community. I recommend a major revision to address the points raised, particularly emphasizing methodological justification, broader implications, and a deeper engagement with the existing body of literature.  
  
Major Comments:  
  
0. The title is a bit long and “different scales” can mean so many different things.

Revised to: “Nitrogen demand, availability, and acquisition strategy control G. max responses to elevated CO2”  
  
1. Originality and Contribution to the Field: the manuscript addresses a crucial aspect of plant ecology and climate research, offering novel insights into how nitrogen availability modulates plant responses to elevated CO2. The integration of eco-evolutionary optimality theory with empirical data enriches the discussion on plant adaptation strategies. However, articulating the study's novelty against the backdrop of existing literature could further highlight its unique contribution. This was done???  
  
2. Methodology and Experimental Design: the detailed description of the experimental setup, including CO2 enrichment, nitrogen fertilization levels, and inoculation treatments, provides a solid foundation for the study. Nonetheless, elaborating on the selection process for the specific levels of nitrogen fertilization and CO2 concentrations could enhance the methodological rigor. Clarifying these choices would help underscore the relevance of the experimental conditions to natural settings and agricultural practices.

Justification for CO2 concentration added. Still need to work through justification for N treatments  
  
3. Results Presentation and Analysis: the results are presented in a clear and structured manner, with statistical analyses robustly supporting the findings. However, further discussion on the biological significance of the observed effects, beyond statistical relevance, would provide a deeper understanding of the implications of these results. Additionally, cross-referencing the supporting information more effectively within the main text could improve the coherence and accessibility of the data presented.  
  
4. Discussion and Interpretation: the discussion adeptly connects the study's findings with broader ecological and evolutionary theories. Yet, a more thorough comparison with conflicting or supporting studies would enrich the narrative, offering a broader perspective on the study's implications within the current scientific discourse. Additionally, expanding on the potential agricultural and ecological applications of the findings could amplify the manuscript's impact.  
  
5. Limitations and Future Directions: the authors acknowledge limitations, which is commendable. Expanding to include potential methodological constraints and the implications of these limitations on the study's findings (including for climate modeling) would reinforce the manuscript's scientific merit. Furthermore, proposing specific future research directions based on the study's outcomes could stimulate ongoing inquiry into this vital area of research.  
  
Minor Comments:  
  
1. Figures and Tables: although figures show different things, they are rather repetitive in style. A more creative visual way to convey the main messages of the paper should be considered.  
  
2. Technical and Editorial Quality: the manuscript is well-written with minor errors. A proofreading session is recommended to correct these minor issues, ensuring the manuscript's professional presentation.  
  
Specific comments:  
  
Line 25: Point one of summary is more of a general hypothesis than an actual finding of the current study.  
  
Line 44: which scales?  
  
Line 58: Not exclusively.  
  
Line 133: See also global studies using the FUN model:  
Fisher, J. B., Sitch, S., Malhi, Y., Fisher, R. A., Huntingford, C., & Tan, S.-Y. (2010). Carbon cost of plant nitrogen acquisition: A mechanistic, globally applicable model of plant nitrogen uptake, retranslocation, and fixation. Global Biogeochemical Cycles, 24(1), n/a-n/a. <https://nam04.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1029%2F2009GB003621&data=05%7C02%7Cevan.a.perkowski%40ttu.edu%7C943f6858a21048ed67b008dc37c3a252%7C178a51bf8b2049ffb65556245d5c173c%7C0%7C0%7C638446560599302745%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=ruu0t6Z0z%2FYDb1SDd7a3y1XzWsi3POxgWWaVEaAPODM%3D&reserved=0>  
  
Shi, M., Fisher, J. B., Brzostek, E. R., & Phillips, R. P. (2016). Carbon cost of plant nitrogen acquisition: global carbon cycle impact from an improved plant nitrogen cycle in the Community Land Model. Global Change Biology, 22(3), 1299–1314. <https://nam04.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1111%2FGCB.13131&data=05%7C02%7Cevan.a.perkowski%40ttu.edu%7C943f6858a21048ed67b008dc37c3a252%7C178a51bf8b2049ffb65556245d5c173c%7C0%7C0%7C638446560599307068%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=uvbTCG7g%2BV266SIIKUU2Um45WHmgPQtYAEFPwYRAZuo%3D&reserved=0>  
  
Braghiere, R. K., Fisher, J. B., Fisher, R. A., Shi, M., Steidinger, B. S., Sulman, B. N., Soudzilovskaia, N. A., Yang, X., Liang, J., Peay, K. G., Crowther, T. W., & Phillips, R. P. (2021). Mycorrhizal Distributions Impact Global Patterns of Carbon and Nutrient Cycling. Geophysical Research Letters, 48(19). <https://nam04.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1029%2F2021GL094514&data=05%7C02%7Cevan.a.perkowski%40ttu.edu%7C943f6858a21048ed67b008dc37c3a252%7C178a51bf8b2049ffb65556245d5c173c%7C0%7C0%7C638446560599311126%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=U3IcTVObLF5i8eH77EssHfxx7jcHpdq27SxO%2B%2BEkl3w%3D&reserved=0>  
  
Braghiere, R. K., Fisher, J. B., Allen, K., Brzostek, E., Shi, M., Yang, X., Ricciuto, D. M., Fisher, R. A., Zhu, Q., & Phillips, R. P. (2022). Modeling global carbon costs of plant nitrogen and phosphorus acquisition. Journal of Advances in Modeling Earth Systems, e2022MS003204. <https://nam04.safelinks.protection.outlook.com/?url=https%3A%2F%2Fdoi.org%2F10.1029%2F2022MS003204&data=05%7C02%7Cevan.a.perkowski%40ttu.edu%7C943f6858a21048ed67b008dc37c3a252%7C178a51bf8b2049ffb65556245d5c173c%7C0%7C0%7C638446560599315037%7CUnknown%7CTWFpbGZsb3d8eyJWIjoiMC4wLjAwMDAiLCJQIjoiV2luMzIiLCJBTiI6Ik1haWwiLCJXVCI6Mn0%3D%7C0%7C%7C%7C&sdata=GED43mG0G3to7cogXGud4LvlGQnML1o6okP%2F10KegqQ%3D&reserved=0>  
  
Line 164: why glycine max and b. japonicum? No reasoning was given for these choices.  
  
Line 633: “optimally coordinated leaves” what does that mean?  
  
  
Referee: 3  
  
Comments to the Author  
In this manuscript the authors used a growth chamber experiment to grow soybeans in a factorial experiment where they manipulated CO2, N fertilization, and inoculation. They measured leaf traits (including gas exchange rates) to test whether the leaf responses to elevated CO2 followed an eco-evolutionary optimality hypothesis (which would predict a reduction in Vcmax relative to Jmax) while whole-plant responses were explained by the progressive N limitation hypothesis (where positive responses to elevated CO2 may be constrained by N limitation). Overall, they report support for these hypotheses.  
  
Generally, the experiment was well conducted and most of the manuscript is well written. However, the results section is difficult to follow (see major comments). Thus, it was not clear how some response variables and their responses to the experimental manipulations pertained to the hypotheses. That said, most of our comments (we co-reviewed) are relatively minor.  
  
Major comments:  
  
The introduction does a good job of setting up the main question as we understand it: **Are plant responses to N and CO2 best explained by the eco-evolutionary optimality hypothesis at the leaf-level and progressive N limitation at the whole-plant level?** However, the results are difficult to follow because (1) the results section is organized differently than the introduction and specifically, the hypotheses, and because (2) so much information is given in the results section (some of which is more important than others to accepting or rejecting the hypotheses). It is also not clear how some of the response variables and their relationships to eCO2 and N fertilization are expected to change according to the hypotheses (i.e., it is not clear why are you measuring some of these things? Chlorophyll is a good example.). We suggest moving some results to the supplement and organizing the results section differently, so it makes it clearer how the results support or refute the hypotheses. One such strategy may be to remind readers in the results section how different variables should be affected by eCO2 and/or N fertilization if, for example, there was to be support for the eco-evolutionary optimality hypothesis.  
  
How is the independence (of photosynthetic responses to eCO2) to N fertilization and inoculation quantified (hypothesis 1 L153-154 and later (e.g., L 622)? That is, do leaf responses to eCO2 that are independent of N fertilization have slopes that are not significant relative to N fertilization? And similarly, do leaf responses to eCO2 that are independent of inoculation have slopes and intercepts that are not significant relative to inoculation? Figure 2a-d show a positive relationship with N fertilization for uninoculated plants and what looks like a big effect of inoculation at lower N fertilization levels. Please clarify.  
  
A conceptual diagram to show how A-Ci responses are expected to change with eCO2 according to the eco-evolutionary optimality hypothesis may be helpful. This can be contrasted with what would be expected under that progressive N limitation hypothesis.  
  
L278-279: We have some concerns about using the mean 15N value from uninoculated individuals at each N fertilization & CO2 treatment without quantifying the effect of variation across reference plants on the calculated uncertainty in %Ndfa. This is especially important to consider since 15N enriched N fertilizer was not used (which would increase the separation of the two end members: atmosphere and soil).  
  
Minor comments:  
  
Title: Since the experiment consisted only of one species, the title can be made more specific by changing “plant” to “Glycine max”. Done  
  
L80-81: Please provide citations or “plant responses to eCO2 are constrained by nitrogen availability.  
  
L81-82: N limits NPP in many ecosystems, but sometimes does not. We suggest adding “often” between “availability” and “limits” to acknowledge this heterogeneity in N limitation.

Done  
  
L82-84: Would it be more specific to say that eCO2 increases plant N demand, which may lead to greater N limitation without a concurrent increase in N supply?

Yes! Done  
  
L84-87: This sentence is a bit confusing and can probably be worded differently. Our understanding is that more N means a greater response of NPP to eCO2. However, over time if there are no concurrent increases in N supply then NPP responses to eCO2 will dampen. Also, it would be good to specify what is meant by “longer-lived tissues”. Does this include detritus? Or is it only living tissues where N is being stored?  
  
L97: Please be more specific about what is meant by “aboveground conditions”.

Done  
  
L111-114: It may be helpful to list other N investments in leaves broadly (non-Rubisco) and crucially specific to those that relate to the hypotheses (e.g., N used in light capturing pigments).  
  
L119-120: Although this is probably true, we are not sure about the stance of New Phytologist towards claims such as “no studies have…”.

Changed to be a bit less direct  
  
L124: “availability” is misspelled.

Corrected  
  
L126: The use of “demand” is not very specific here. We assume you mean leaf-level demand but not whole-plant-level demand. That said, this sentence can probably be shortened for clarity.

Corrected  
  
L152: Is “equal” needed? It seems like this is implicitly understood from “co-limitation”.  
Corrected

L159-160: This is not as clear as it could be. Can it be stated that the lack of growth differences due to eCO2 and inoculation at higher levels of N fertilization is due to a lack of N limitation to growth? Does “individuals will invest more strongly in symbiotic nitrogen fixation” imply that N fixation should fully down-regulate with N fertilization, or not necessarily?  
Corrected

L263-266: It would be helpful to explain (probably in the introduction) how chlorophyll measurements relate to the hypotheses. E.g., how would a greater decrease in Vcmax than Jmax under the eco-evolutionary optimality hypothesis affect chlorophyll?  
  
L282: Given that there is some N in seeds, inoculum, and the growing medium, using a value of delta15N\_fixation that is from an unfertilized plant may be a bit different than the true isotopic ratio of atmospheric N2. Why not use 0.3663 atom % 15N instead (Dawson et al., 2002, Annual Review of Ecology & Systematics)?

Statistics section: Is there a rationale for not doing model selection (e.g., using AICc) to select the best model? Coefficient values may change for parameters that are significant if additional insignificant parameters are not included.

Yes, our models were formed using *a priori* hypotheses and there is no reason to remove insignificant parameters as these are important patterns that we have verifiable hypotheses for.

Leaf nitrogen content section of Results: Should figure 1 be referenced earlier? It may help readers follow along with the results.  
  
L541, 558, 568: It is probably best to remove “interestingly” here, as that statement is subjective. It can however, where appropriate, be changed to “contrary to our predictions” or something along those lines.

Done  
  
L605-617: This section probably does not belong in the Discussion. It validates the rationale for how the experiment was set up (pot size), but that information would probably be better in the supplement or the methods section.

Removed  
  
Figures: Please state what the shading represents. Is it 95% confidence intervals?

Yes, added  
  
Figures: We believe some of the analyses in the tables addressed whether lines or curves in the figures are significantly different than each other (i.e., the fixed effects in the models), but it is not clear if they are from the figures. For example, we think it would be helpful to know if blue points in Figure 1b are best represented by two lines (and if they are parallel or not), or one line.

This is better explained in the main text of the Results. Any interactions between nitrogen and CO2 are included in the supplemental material.  
  
Figure 3d: It is not clear to us why uninoculated plants are included here, since the purpose of those points is to calculate %Ndfa for the inoculated plants.

Uninoculated plants are included here as a proof-of-concept for the experiment. We can remove these values.